REMARKS

STATUS OF THE CLAIMS:

Claims 1 to 20 are cancelled.

Claims 30, 31, 33, 44, 45, 46, 55 have been amended.

Claims 21 to 55 are pending.

Claim 30 was amended to correct a typographical error by substituting the incorrect dependency from "claim 31" to the correct dependency from "claim 29" to overcome the Examiner's rejection of the same. Support for this amendment may be found on pages 75, 78 to 79, 99, 129, 139 to 141, and Example 19 of the specification, and throughout the application as original filed. Applicants right to equivalents of Claim 30 is reserved. No new matter has been added.

Claim 31 was amended to correct a typographical error by substituting the incorrect dependency from "claim 32" to the correct dependency "claim 30" to overcome the Examiner's rejection of the same. Support for this amendment may be found on pages 75, 78 to 79, 99, 129, 139 to 141, and Example 19 of the specification, and throughout the application as original filed. Applicants right to equivalents of Claim 31 is reserved. No new matter has been added.

Claim 33 was amended to append the phrase "encoding a polypeptide" after the "polynucleotide" term to overcome the Examiner's rejection of the same. Support for this amendment may be found on pages 54 to 56 of the specification. Applicants right to equivalents of Claim 33 is reserved. No new matter has been added.

Claim 44 was amended to substitute the phrase "1 to 2057" with the phrase "-2057 to -1", as well as to substitute the phrase "SEQ ID NO:27" with the phrase "the sequence provided in Figures 7A-B" to reflect the change from positive numbering to negative numbering between SEQ ID NO:27 and the sequence provided in Figures 7A-B, as well as to make the numerical references to the sequence provided in Figures 7A-B (SEQ ID NO:27) consonant with the numerical references to the CpG island locations in the amended dependent claims 45 and 46. Applicants note this change was warranted on account of negative numbering not being permitted for nucleotide sequences in Sequence Listings in accordance with WIPO Standard

ST.25. Rather, all nucleotide sequences must begin with number 1 (See 37 CFR § 1.822 (c)(6)). Support for these amendments may be found in Figures 7A-B of the specification. Applicants right to equivalents of Claim 44 is reserved. No new matter has been added.

Claim 45 was amended to append the "but not more than 10" phrase after the "at least one nucleotide" phrase; and to substitute the phrase "within the CpG island regions encompassed by nucleotides 90 to 312, 836 to 1122, or 1331 to 1589" with the phrase "are mutated in a region selected from the group consisting of: a.) from about nucleotide –1968 to about –1746; b.) from about nucleotide –1232 to about –936; and c.) from about nucleotide –727 to about –470". Support for these amendments may be found in the legend of Figures 7A-B on page 14, and pages 27 to 28, and in the paragraph beginning on page 66, line 26. Claim 45 was further amended to delete the phrase "of SEQ ID NO:27 are mutated"; to append the term ", wherein" after the newly added "–470" term; to delete the phrase "such that " after the newly added ", wherein" term; to substitute the phrase "the CpG islands contained therein" with the phrase "said regions"; and to delete the phrase "capable of being" in an effort to increase the clarity of this claim. Applicants right to equivalents of Claim 45 is reserved. No new matter has been added.

Claim 46 was amended to substitute the phrase "at least one CpG island region encompassed by nucleotides 90 to 312, 836 to 1122, and 1331 to 1589" with the phrase "a region selected from the group consisting of: a.) from about nucleotide –1968 to about –1746; b.) from about nucleotide –1232 to about –936; and c.) from about nucleotide –727 to about –470" to overcome the Examiner's objection to the same. Support for this amendment may be found in the legend of Figures 7A-B on page 14, and pages 27 to 28. Claim 46 was further amended to delete the phrase "of SEQ ID NO:27". Applicants right to equivalents of Claim 46 is reserved. No new matter has been added.

Claim 55 was amended to delete the phrase "; and wherein said polynucleotide does not hybridize under stringent conditions to a nucleic acid molecule having a nucleotide sequence of only A residues or of only T residues" phrase. Support for this amendment may be found in the paragraph beginning on page 3, line 25. Claim 55 was further amended to substitute the phrase "provided in Claim 1" with the phrase "encoding amino acids 2 to 541 of SEQ ID NO:2". Support for this amendment may be found in Claim 21. Applicants right to equivalents of Claim 55 is reserved. No new matter has been added.

I. Miscellaneous

a. Public Access to ATCC Deposit No. PTA-4454

Applicants representative hereby gives the following assurance by signature below:

Bristol-Myers Squibb Company, an assignee of the present application, has deposited biological material under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure with the following International Depository Authority: American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Virginia 20110-2209. This deposit comprises the cDNA sequences of BGS-42 clone A, BGS-42 clone B, and BGS-42 clone C that together encode the full-length BGS-42 polypeptide of the present invention. The deposit for BGS-42 was made on June 12, 2002, and given ATCC Accession Number PTA-4454. In accordance with MPEP 2410.01 and 37 C.F.R. § 1.808, assurance is hereby given that all restrictions on the availability to the public of ATCC Accession Number PTA-4454 will be irrevocably removed upon the grant of a patent based on the captioned application, except as permitted under 37 C.F.R. § 1.808(b).

Applicants representative also hereby gives the following additional assurance by signature below:

In accordance with 37 C.F.R. § 1.805 to § 1.807, assurance is hereby given that the viability of the deposits for BGS-42 clone A, BGS-42 clone B, and BGS-42 clone C, made on June 12, 2002, and given ATCC Accession Number PTA-4454, will be maintained during the pendency of the captioned application for a duration of at least 30 years or at least five years after the most recent request for the furnishing of a sample of the deposit is received by the ATCC; and that the deposit will be replaced if it should ever become inviable.

A copy of the ATCC Deposit receipt for Accession Number PTA-4454 was provided with Applicants September 1, 2005 Preliminary Amendment.

II. Rejections under 35 U.S.C. § 112, first paragraph

a. The Examiner has rejected Claims 32-35, 39-43 and 53-54 under 35 U.S.C. § 112, first paragraph, alleging that these claims fail to comply with the enablement requirement and contain subject matter which was not described in the specification in such a way as to enable

one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. More particularly, the Examiner alleges

> Claims 32-35, 39-43 and 53-54 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for isolated nucleic acids encoding BGS42, does not reasonably provide enablement for any of the following:
> (a) isolated DNA sequences comprising polynucleotides having 80% identity to those encoding residues 1-541 or 2-541 of SEQ ID NO:2, with no function.
> (b) isolated DNA sequences encoding a polypeptide comprising 50 or 394 contiguous amino acids of SEQ ID NO:2 with no function.
> (c) isolated nucleic acid molecules comprising 150 contiguous nucleotides of SEQ ID NO:l, with no function.
> (d) isolated DNA sequences comprising residues 369-1247 and 549-1274 of SEQ ID NO:I or encoding residues 73-365 of SEQ ID NO:2, with no function. (e) an isolated polynucleotide that hybridizes to that encoding residues1-541 or 2-541 of SEQ ID NO:2, under stringent conditions recited in claim 55, and wherein said polynucleotide does not hybridize to a nucleotide sequence of only A or T residues (see claim 55), with no function.
>
> The criteria for undue experimentation, summarized in *re Wands,* 8, USPQ2n 1400 (Fed. Cir. 1988) are: 1) the quantity of experimentation necessary, 2) the amount of direction or guidance presented, 3) the presence and absence of working examples, 4) the nature of the invention, 5) the state of prior art, 6) the relative skill of those in the art, 7) the predictability or unpredictability of the art, and 8) the breadth of the claims.
>
> The specification fails to teach which residues within the claimed DNA sequences should remain intact such that said products listed as (a)-(e) above retain their function. No examples of such critical residues are provided either. Current state of the art indicates that once more than 3-9 residues of a polynucleotide encoding a full-length polypeptide is simultaneously mutated (see parts (a) and (e) above) or once most of the structure of a polynucleotide encoding a full-length polypeptide is mutated such that it only needs to either encode a product comprising 50 or 394 residues of a full- length polypeptide (see part (b)) or needs to merely comprise a region encoding fragments of said full-length polypeptide (see parts (c)-(d)), said product is not necessarily capable of encoding products with the same activity as said full-length polypeptide.
>
> Therefore due to lack of sufficient guidance and examples provided in the specification and due to unpredictability of prior art as to which residues within the polynucleotides of parts (a)-(e) above should be retained such that said products encode products with tyrosine ligase activity one of skill in the art has to go through the burden of undue experimentation in order to screen for those products that are within he scope of this invention and as such the claims go beyond the scope of the disclosure.

Applicants disagree with the Examiner's allegation and assert that one skilled in the art could make and use the invention embraced by Claims 32 to 35, 39 to 43 and 53 to 54. Relative to Claims 34 to 35, 39 to 42, and 53 to 54, Applicants point out to the Examiner that a skilled artisan would only require knowledge of the sequence encompassed by these claims to actually make and use the invention. First, Applicants assert that one skilled in the art could easily make the invention of Claims 34 to 35, 39 to 42, and 53 to 54 based upon the teachings of Applicants specification since the entire polynucleotide and polypeptide sequence of BGS-42 is disclosed and could easily be used as a basis for making a sequence that encodes at least 50 contiguous amino acids of SEQ ID NO:2, a polynucleotide encoding amino acids 73 to 365 of SEQ ID NO:2, a polynucleotide encoding amino acids 133 to 374 of SEQ ID NO:2, and a polynucleotide encoding at least 394 contiguous amino acids of SEQ ID NO:2 using methods well-known in the art and described in Applicants specification. Specifically, PCR methodology is mature in the art and the skilled artisan could readily design primers that could be used to clone any such fragment simply using the BGS-42 sequence as a template. Such methods are explicitly described in Example 18 of the instant specification. Moreover, Applicants specification also explicitly discloses each of these sequences individually, in addition to many other species that are specifically encompassed by the language of the claims directed to the same. Specifically, Applicants specification discloses all N- and C-terminal deletion mutants of the BGS-42 polypeptide, their encoding polynucleotides (see pages 35 to 41), in addition to providing explicit teachings as to how one skilled in the art could actually make these mutants (see Example 18). As a consequence, the instant specification explicitly discloses 491 N-terminal deletion mutants that comprise "at least 50 contiguous amino acids of SEQ ID NO:2" (e.g., M1-S541 to D491-S541 on pages 35 to 38) and explicitly discloses 491 C-terminal deletion mutants that comprise "at least 50 contiguous amino acids of SEQ ID NO:2" (e.g., M1-L51 to M1-S541 on pages 38 to 40) along with their encoding polynucleotides. Clearly one skilled in the art could make such polynucleotides based upon the extensive teachings of Applicants specification.

Applicants specification also discloses amino acids 73 to 365 of SEQ ID NO:2 in Example 1, in Figures 1A-C, in Figure 4, in Claim 1, on page 8, as well as its encoding polynucleotides in Figures 1A-C. Applicants remind the Examiner that the M.P.E.P. does not require the explicit listing of each sub-sequence in the Sequence Listing, rather reference to the nucleotides of a sequence in the Sequence Listing is sufficient. (see M.P.E.P. 2422.03). As

discussed *supra*, the mere disclosure of such a sequence is sufficient to enable one skilled in the art to actually make and clone the sequence considering the maturity of PCR cloning methodology. Nonetheless, a polypeptide comprising amino acids 73 to 365 of SEQ ID NO:2 may also be obtained by combining the E73-S541 N-terminal deletion mutant on page 36 with the M1-I365 C-terminal deletion mutant on page 39 and following the cloning methods described in Example 18. The combination of N- and C-terminal deletion mutants is specifically disclosed in the paragraph beginning on page 40, line 31 and continuing onto to page 41.

Applicants specification also discloses amino acids 133 to 374 of SEQ ID NO:2 in Figure 2. Figure 2 discloses 242 amino acids of BGS-42 (SEQ ID NO:4) and its encoding polynucleotide sequence (SEQ ID NO:3). Amino acids 133 to 374 of SEQ ID NO:2 directly correspond to the 242 amino acids of SEQ ID NO:4 (see Exhibit A submitted concurrently herewith). As discussed *supra*, the mere disclosure of such a sequence is sufficient to enable one skilled in the art to actually make and clone the sequence considering the maturity of PCR cloning methodology. Nonetheless, a polypeptide comprising amino acids 133 to 374 of SEQ ID NO:2 may also be obtained by combining the I133-S541 N-terminal deletion mutant on page 36 with the M1-Q374 C-terminal deletion mutant on page 39 and following the cloning methods described in Example 18. The combination of N- and C-terminal deletion mutants is specifically disclosed in the paragraph beginning on page 40, line 31 and continuing onto to page 41.

Applicants specification also discloses polynucleotides encoding at least 394 contiguous amino acids of SEQ ID NO:2 by explicitly disclosing 148 N-terminal deletion mutants that comprise "at least 394 contiguous amino acids of SEQ ID NO:2" (e.g., M1-S541 to S148-S541 on pages 35 to 36) and explicitly discloses 148 C-terminal deletion mutants that comprise "at least 394 contiguous amino acids of SEQ ID NO:2" (e.g., M1-V394 to M1-S541 on pages 38 to 39) along with their encoding polynucleotides. As discussed *supra*, the mere disclosure of such a sequence is sufficient to enable one skilled in the art to actually make and clone the sequence considering the maturity of PCR cloning methodology. Nonetheless, a polypeptide comprising at least 394 amino acids of SEQ ID NO:2 may be obtained following the cloning methods described in Example 18.

Clearly, one skilled in the art could readily make such polynucleotides based upon the extensive teachings of Applicants specification.

Regarding use of the polynucleotides encompassed by Claims 32 to 35, 39 to 43 and 53 to 54, Applicants point out to the Examiner that one of the disclosed and supported utilities for the BGS-42 polynucleotides is their use in methods of diagnosing testicular cancer (see Figure 11, pages 26 and 27, and Example 6). As the Examiner will appreciate, it is well established in the art that small fragments of a polynucleotide sequence can be used as probes for identifying and quantifying expression levels of a gene in any given sample. For example, the oligonucleotides directed to the BGS-42 polynucleotide originally used to associate BGS-42 to testicular cancer were only 21, 20, and 26 nucleotides in length (see Example 6; and SEQ ID NOS:28, 29, and 30, respectively). Clearly, the skilled artisan would recognize that any fragment longer than this length, let alone one that is at least 7.5 times this length (e.g., a polynucleotide encoding at least 50 amino acids would be 150 nucleotides in length which is 7.5x longer than the 20mer provided as SEQ ID NO:29) would be useful in measuring the expression levels of the BGS-42 polynucleotide, and hence useful for diagnosing testicular cancer. The sequences encompassed by Claims 34 to 35, 39 to 42, and 53 to 54 are at least 150 nucleotides in length, with Claims 39 to 40, 41 to 42, and 53 to 54 being directed to polynucleotides that are over 43 fold (293 amino acids encoded by 879 nucleotides divided by 20 nucleotides equals 43.95), over 36 fold (242 amino acids encoded by 726 nucleotides divided by 20 nucleotides equals 36.3), and over 59 fold (394 amino acids encoded by 1182 nucleotides divided by 20 nucleotides equals 59.1) longer than the 20 nucleotide primer used to measure the expression level of BGS-42.

Accordingly, Applicants assert that the instant disclosure clearly satisfies the enablement requirement for Claims 34 to 35, 39 to 42, and 53 to 54 since: (i) no additional experimentation is necessary on account of the extensive teachings of Applicants specification demonstrating explicit use of fragments significantly smaller than the fragments encompassed by these claims as well as explicit teachings of species encompassed by the same; (ii) the extensive amount of direction or guidance presented; (iii) the existence of working examples using fragments significantly shorter than the fragments encompassed by these claims (e.g., Example 6); (iv) the nature of the invention and its use in diagnostic methods; (v) the mature state of the diagnostic arts; (vi) the mature skill of those in the art in making and using gene fragments for diagnostic utility; (vii) the high level of predictability in the diagnostic arts; and (viii) the reasonable breadth of the claims.

Applicants believe the enablement requirement has been met and that the Examiners rejection of Claims 34 to 35, 39 to 42, and 53 to 54, has been overcome and respectfully request that the Examiner withdraw the same.

Relative to Claims 32 to 33, Applicants point out to the Examiner that one skilled in the art could easily make and use the invention of these claims based upon the teachings of Applicants specification since the instant specification explicitly discloses a significant number of sequences that are encompassed by the "at least 80.0%" identity limitation of these claims. Specifically, the instant specification explicitly discloses all N- and C-terminal deletion mutants of the BGS-42 polypeptide, their encoding polynucleotides (see pages 35 to 41), in addition to providing explicit teachings as to how one skilled in the art could actually make these mutants (see Example 6). Applicants specification explicitly discloses over 109 N-terminal deletion mutants and over 109 C-terminal deletion mutants individual species, respectively, embraced by Claims 32 to 33 (433 amino acids divided by 540 amino acids (i.e., amino acids 2 to 541 are 540 amino acids) equals 80.18%; and there are 109 individual deletion species beginning from N109-S541 to M1-S541 on pages 35 to 36; and there are 109 individual deletion species beginning from M1-G433 to M1-S541 on pages 38 to 39). Applicants bring to the attention of the Examiner a sequence alignment demonstrating that sequences at least 433 contiguous amino acids in length are "at least 80.0%" identical to SEQ ID NO:2 (see Exhibit B-1 and B-2) using a CLUSTALW global sequence alignment algorithm with default parameters (as provided with the AlignX software program of the Vector NTI suite of programs, version 6.0) as specified in Claims 32 to 33. Exhibit B-1 provides the sequence alignment between the full-length BGS-42 polypeptide sequence with a portion of BGS-42 that is 433 amino acids in length. Exhibit B-2 provides a screen shot of the alignment between these two sequences along with the setup window demonstrating that default parameters were used as specified in Claims 32 and 33 and delineated on page 56 of the specification. Calculations have not been provided for polynucleotides, however, the results are the same (433 amino acids encoded by 1299 nucleotides divided by 540 amino acids encoded by 1620 nucleotides equals 80.18%). Clearly, one skilled in the art could readily make a polynucleotide embraced by these claims based upon the extensive teachings of Applicants specification and the fact that more than a representative number of species embraced by these claims are disclosed in addition to providing explicit teachings as to how one skilled in the art could actually make these mutants.

Regarding use of the polynucleotides encompassed by Claims 32 to 33, Applicants remind the Examiner that one of the utilities for the BGS-42 polynucleotides is for use in methods of diagnosing testicular cancer (see *supra*). Consequently, it is well established in the art that fragments of a polynucleotide sequence can be used as probes for identifying and quantifying expression levels of the same in any given sample. As exemplified in Exhibits B-1 and B-2, Applicants specification explicitly discloses a significant number of fragments of BGS-42 that are longer than 433 amino acids in length and encompassed by the "at least 80.0%" limitation of these claims. Furthermore, as discussed *supra*, the oligonucleotides directed to the BGS-42 polynucleotide originally used to associate BGS-42 to testicular cancer were as short as 20 nucleotides in length (see Example 6; and SEQ ID NO:29). Clearly, the skilled artisan would recognize that a fragment over 64 times this length (e.g., 433 amino acids encoded by 1299 nucleotides divided by 20 nucleotides equals 64.95) would be useful in measuring the expression levels of the BGS-42 polynucleotide, and hence useful for diagnosing testicular cancer.

Accordingly, Applicants assert that the instant disclosure clearly satisfies the enablement requirement for Claims 32 to 33 since: (i) no additional experimentation is necessary on account of the extensive teachings of Applicants specification demonstrating explicit use of fragments significantly smaller than the fragments encompassed by these claims as well as explicit teachings of species encompassed by the same; (ii) the extensive amount of direction or guidance presented; (iii) the existence of working examples using fragments significantly shorter than the fragments encompassed by these claims (e.g., Example 6); (iv) the nature of the invention and its use in diagnostic methods; (v) the mature state of the diagnostic arts; (vi) the mature skill of those in the art in making and using gene fragments for diagnostic utility; (vii) the high level of predictability in the diagnostic arts; and (viii) the reasonable breadth of the claims.

Applicants also disagree with the Examiners allegation that a "functional limitation or any particular conserved structure" is required to be recited in the claims since, as discussed *supra*, one skilled in the art would know how to make and use the invention for its use in diagnosing testicular cancer and the fact that such a use is not dependent upon the functional activity of the BGS-42 polypeptide.

Regarding Claim 43, Applicants believe the Examiner's rejection of this claim is in error since Claim 43 is directed to the complementary sequence of the sequences encompassed by

Claim 21 and the Examiner does not recite any basis for rejecting the complementary sequence of those sequences under 35 U.S.C. § 112, first paragraph.

Applicants believe the enablement requirement has been met and that the Examiners rejection of Claims 32 to 35, 39 to 43 and 53 to 54 under 35 U.S.C. § 112, first paragraph, has been overcome and respectfully request that the Examiner withdraw the same.

b. The Examiner has rejected Claims 36 to 38 under 35 U.S.C. § 112, first paragraph, alleging that these claims fail to comply with the enablement requirement. More particularly, the Examiner alleges

> With respect to claims 36-38, It is noted that the applicants have deposited the organisms under the terms of Budapest treaty but there is no indication in the specification as to the public availability. Since the deposit was made under the terms of the Budapest Treaty, then an affidavit or declaration by applicants, or a statement by an attorney of record over his or her signature and registration number, stating that the specific strain has been deposited under the Budapest Treaty and that the strain will be irrevocably and without restriction or condition released to the public upon the issuance of the patent, would satisfy the deposit requirement made herein.

In response, Applicants representative has provided the required assurance in the "Miscellaneous" section of Applicants Reply *supra*. Applicants believe the Examiners rejection of Claims 36 to 38 under 35 U.S.C. § 112, first paragraph has been overcome in consideration of Applicants assurances provided herein.

III. Rejections under 35 U.S.C. § 112, first paragraph

a. The Examiner has rejected Claims 32-35, 39-43 and 53-54 under 35 U.S.C. § 112, first paragraph, alleging that these claims fail to comply with the written description requirement and contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. More particularly, the Examiner alleges

> Claims 32-35, 36-38,39-43, 53-54 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in

such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. Claims 32-35, 39-43 and 53-54 are directed to a genera of polynucleotides that have been inadequately described in the specification:

(a) a genus of isolated DNA sequences comprising polynucleotide having 80% identity to those encoding residues 1-541 or 2-541 of SEQ ID NO:2, with no function.

(b) a genus of isolated DNA sequences encoding a polypeptide comprising 50 or 394 contiguous amino acids of SEQ ID NO:2 with no function.

(c) a genus of isolated nucleic acid molecules comprising 150 contiguous nucleotides of SEQ ID NO:1, with no function.

(d) a genus of isolated DNA sequences comprising residues 369-1247 and 549-1274 of SEQ ID NO:I or encoding residues 73-365 of SEQ ID NO:2, with no function.

(e) a genus of isolated polynucleotides that hybridize to that encoding residues either 1-541 or 2-541 of SEQ ID NO:2, under stringent conditions recited in claim 55, and wherein said polynucleotides do not hybridize to a nucleotide sequence of only A or T residues (see claim 55), with no function.

The specification does not contain any disclosure of the function of all DNA sequences that are listed in sections (a)-(e) above. The genus of cDNAs that comprise these above cDNA molecules is a large variable genus with the potentiality of encoding many different proteins. Therefore, many functionally unrelated DNAs are encompassed within the scope of these claims, including partial DNA sequences. The specification discloses only a single species (namely polynucleotides encoding SEQ ID NO:2) of the claimed genus which is insufficient to put one of skill in the art in possession of the attributes and features of all species within the claimed genus. Therefore, one skilled in the art cannot reasonably conclude that the applicant had possession of the claimed invention at the time the instant application was filed.

Applicants disagree and point out that the instant specification does, in fact, provide adequate teachings to reasonably convey to a skilled artisan that Applicants were in possession of the polynucleotides embraced by Claims 32-35, 39-43 and 53-54 as demonstrated *supra* in section II. Specifically, Applicants specification explicitly discloses 491 N-terminal deletion mutants that comprise "at least 50 contiguous amino acids of SEQ ID NO:2" (e.g., M1-S541 to D491-S541 on pages 35 to 38) and explicitly discloses 491 C-terminal deletion mutants that comprise "at least 50 contiguous amino acids of SEQ ID NO:2" (e.g., M1-L51 to M1-S541 on pages 38 to 40) along with their encoding polynucleotides. Applicants specification explicitly discloses 148 N-terminal deletion mutants that comprise "at least 394 contiguous amino acids of SEQ ID NO:2" (e.g., M1-S541 to S148-S541 on pages 35 to 36) and explicitly discloses 148 C-terminal deletion mutants that comprise "at least 394 contiguous amino acids of SEQ ID NO:2"

(e.g., M1-V394 to M1-S541 on pages 38 to 39) along with their encoding polynucleotides. Applicants point out disclosure of a representative number of species is all that is required to satisfy the written description requirement of a genus claim. Accordingly, Applicants assert one skilled in the art would recognize that one skilled in the art would recognize Applicants were in possession of the genus encompassing polypeptides comprising at least 50 contiguous amino acids of SEQ ID NO:2, at least 150 contiguous nucleotides of SEQ ID NO:1, and at least 394 contiguous amino acids of SEQ ID NO:2 based upon the extensive disclosure of numerous species meeting these limitations in the application as originally filed.

The specification explicitly discloses amino acids 73 to 365 of SEQ ID NO:2 in Example 1, in Figures 1A-C, in Figure 4, in Claim 1, on page 8, as well as its encoding polynucleotides in Figures 1A-C. Accordingly, Applicants assert one skilled in the art would recognize that one skilled in the art would recognize Applicants were in possession of this sequence based upon the explicit disclosure of this sequence in the application as originally filed.

Additionally, the specification also explicitly discloses over 109 N-terminal deletion mutants and over 109 C-terminal deletion mutants individual species, respectively, embraced by Claims 32 to 33 as discussed *supra*. Applicants point out disclosure of a representative number of species is all that is required to satisfy the written description requirement of a genus claim. Accordingly, Applicants assert one skilled in the art would recognize that one skilled in the art would recognize Applicants were in possession of the genus encompassing polypeptides encoding polypeptides comprising at least 80% identity to amino acids 2 to 541 of SEQ ID NO:2 and polynucleotides comprising at least 80% identity with the polynucleotides encompassed by Claims 21, based upon the extensive disclosure of numerous species meeting these limitations in the application as originally filed.

Applicants also point out that each of the species discussed *supra* would be expected to hybridize to either a polynucleotide encoding amino acids 1 to 541 or 2 to 541 of SEQ ID NO:2 and thus constitute a representative number of species. Accordingly, the instant specification does in fact disclose a representative number of species to define the genus encompassed by Claim 55 and to convey to one skilled in the art that Applicants were in possession of the claimed genus.

In view of the foregoing, Applicants disagree with the Examiner's allegation that the specification "discloses only a single species (namely polynucleotides encoding SEQ ID NO:2)".

Regarding Claim 43, Applicants believe the Examiner's rejection of this claim is in error since Claim 43 is directed to the complementary sequence of the sequences encompassed by Claim 21 and the Examiner does not recite any basis for rejecting the complementary sequence of those sequences under 35 U.S.C. § 112, first paragraph.

Applicants believe the Examiner's rejection of Claims 32-35, 39-43 and 53-54 under 35 U.S.C. § 112, first paragraph has been overcome and respectfully request that the Examiner withdraw this rejection in consideration of the arguments presented herein.

b. The Examiner has rejected Claims 39 to 40, 45 to 46, and 53 to 54 under 35 U.S.C. § 112, first paragraph, alleging that these claims fail to comply with the written description requirement and contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. More particularly, the Examiner alleges

> The examiner searched the specification for the support of isolated nucleic acid molecules encoding SEQ ID NO:2 attached to mutants (including deletion mutants) of a specific promoter set forth as SEQ ID NO:27 in the regions indicated in claims 45-46 as well support for the subject matter of claims 53-54, 39-40 and could not find any. Hence, for examination purposes said claims are considered to be New Matter. Applicant is advised to either refer the examiner to the places in the specification wherein support for said claims are provided or possibly delete said claims.

Applicants disagree with the Examiner's allegation and assert that the subject matter encompassed by Claims 39 to 40, 45 to 46, and 53 to 54 is disclosed in the specification as originally filed. Specifically, Claims 39 and 40 are directed to a "polynucleotide encoding a polypeptide comprising amino acids 73 to 365 of SEQ ID NO:2". As discussed *supra*, the specification discloses amino acids 73 to 365 of SEQ ID NO:2 in Example 1, in Figures 1A-C, in Figure 4, in Claim 1, on page 8, as well as its encoding polynucleotides in Figures 1A-C. Accordingly, Applicants believe the Examiner's rejection of Claims 39 and 40 under 35 U.S.C. § 112, first paragraph alleging they contain new matter is in error and respectfully the rejection be withdrawn.

Relative to Claims 45 to 46, these claims are directed to either the mutation of or deletion of specific CpG island regions within the BGS-42 promoter region. As Applicants discussed

supra, negative numbering is not permitted for polynucleotide sequences within a sequence listing. The latter posed a challenge in drafting a claim directed to the CpG islands since the location of these islands are expressed with negative numbers in the specification (see Figures 7A-B, the legend for Figures 7A-B on page 14, and on pages 27 to 28). Since the BGS-42 promoter sequence is in the Sequence Listing (SEQ ID NO:27), and since the other claims refer to sequences via their assigned SEQ ID NO:s, the location of the CpG island regions were redefined in Claims 45 and 46 to correspond to the positive number locations of the same in SEQ ID NO:27. The latter resulted in changing the locations from nucleotide –1968 to –1746, nucleotide –1232 to –936, or nucleotide –727 to –470 (i.e., as defined in Figures 7A-B) to nucleotides 90 to 312, 836 to 1122, and 1331 to 1589 of SEQ ID NO:27. Nonetheless, Applicants have amended Claims 45 and 46 to recite the original negative numbers for the CpG island locations and changed the reference sequence from SEQ ID NO:27 to the sequence provided in Figures 7A-B. Accordingly, Applicants believe the Examiner's rejection of Claims 45 and 46 under 35 U.S.C. § 112, first paragraph alleging they contain new matter is in error, but nonetheless has been overcome in consideration of Applicants amendments, and respectfully request that the rejection be withdrawn.

Relative to Claims 53 and 54, Applicants disagree with the Examiner's allegation and assert that the subject matter encompassed by these claims is disclosed in the specification as originally filed. Specifically, Claims 53 and 54 are directed to a "polynucleotide encoding a polypeptide comprising at least 394 contiguous amino acids of SEQ ID NO:2". As discussed *supra*, the specification discloses such sequences by explicitly disclosing 148 N-terminal deletion mutants that comprise "at least 394 contiguous amino acids of SEQ ID NO:2" (e.g., M1-S541 to S148-S541 on pages 35 to 36) and explicitly discloses 148 C-terminal deletion mutants that comprise "at least 394 contiguous amino acids of SEQ ID NO:2" (e.g., M1-V394 to M1-S541 on pages 38 to 39) along with their encoding polynucleotides. As a consequence, the phrase "at least 394 contiguous amino acids of SEQ ID NO:2" is representative of the genus embraced by these deletion mutants, and since each of the species comprising this genus are explicitly disclosed, the genus representative of these species is also explicitly disclosed.

The Examiner appears to imply that in order for a claim limitation to satisfy the written description requirement, such a limitation must be explicitly disclosed in the specification. However, the latter is not consistent with the M.P.E.P., nor judicial precedent. The M.P.E.P.

states that claim limitations may be supported in the specification through "express, implicit, or inherent disclosure..." and that "there is no *in haec verba* requirement" (see M.P.E.P. 2163(I)(B))(emphasis added). Rather, the M.P.E.P. teaches that whether the written description requirement is met turns on whether "...a skilled artisan would have understood the inventor to be in possession of the claimed invention at the time of filing, even if every nuance of the claims is not explicitly described in the specification...See, e.g., Vas-Cath, 935 F.2d at 1563, 19 USPQ2d at 1116; Martin v. Johnson, 454 F.2d 746, 751, USPQ 391, 395 (CCPA 1972)(stating "the description need not be in *ipsis verbis* [i.e., "in the same words"] to be sufficient"). (see M.P.E.P. 2163(II)(A)(3)(a))(emphasis added). For claims directed to a genus, the written description requirement "for a claimed genus may be satisfied through sufficient description of a representative number of species" and "[s]atisfactory disclosure of a 'representative number' depends on whether one of skill in the art would recognize that the applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus in view of the species disclosed", and that "[d]escription of a representative number of species does not require the description to be of such specificity that it would provide individual support for each species that the genus embraces" (see MPEP2163(II)(A)(3)(a)(ii)).

Accordingly, Applicants assert that a genus claim encompassing a representative number of species that are explicitly disclosed in the specification, complies with the written description requirement since one skilled in the art would have appreciated Applicants were in possession of the genus based upon the explicit disclosure of over 296 individual species, and believe the Examiner's rejection of Claims 53 and 54 under 35 U.S.C. § 112, first paragraph alleging they contain new matter is in error, and respectfully request that the rejection be withdrawn.

III. Rejections under 35 U.S.C. § 112, second paragraph

a. The Examiner has rejected Claim 55 under 35 U.S.C. § 112, second paragraph alleging that it is indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. Specifically, the Examiner alleges

> "The phrase 'does not hybridize under stringent conditions to a nucleic acid molecule having a nucleotide sequence of only A residues or of only T residues' is unclear. It is unclear whether applicant is referring to a nucleic acid that is

completely made up of A or T residues or other residues should also be present. Appropriate clarification is required."

Applicants disagree with the Examiner's allegation. However, in the sole interest of facilitating prosecution, Applicants have amended Claim 55 to delete the phrase "; and wherein said polynucleotide does not hybridize under stringent conditions to a nucleic acid molecule having a nucleotide sequence of only A residues or of only T residues". Applicants point out this phrase is an unnecessary limitation since one skilled in the art would recognize that the polynucleotide encoding amino acids 2 to 541 does not contain any poly-A or poly-T stretches of nucleotides, rather it is directed to the polynucleotides encoding the specified amino acids within the coding region of BGS-42, and thus would clearly not be expected to hybridize to complementary sequences containing such poly-A or poly-T nucleotide stretches under stringent hybridization conditions. Accordingly, Applicants believe the Examiner's rejection of Claim 55 under 35 U.S.C. § 112 second paragraph, has been overcome in consideration of this amendment.

b. The Examiner has rejected Claims 45 to 46 under 35 U.S.C. § 112, second paragraph alleging that it is indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. Specifically, the Examiner alleges "[t]he term "CpG island regions" in claim 45 and 46 is unclear."

Applicants disagree with the Examiner's assertion and point out that the phrase "CpG island" is well-known in the art and is recognized as being sites of hypermethylation in the promoter regions of specific genes associated with cancer (see Abstract and p.688 of the Introduction of Baylin et al., Hum. Mol. Genet., 10(7):687-692 (2001) submitted concurrently herewith for the convenience of the Examiner). However, in the sole interest of facilitating prosecution, Applicants have deleted the "CpG island regions" phrase.

The Examiner has further rejected Claim 45 alleging "[t]he phrase 'at least one nucleotide within the CpG island regions encompassed by nucleotides ...' is unclear. How could one nucleotide be encompassed by many? Also, the entire claim 45 does not make grammatical sense. Appropriate correction is required."

Applicants disagree with the Examiner's allegation and point out that the "encompassed by" phrase was necessarily directed to the "CpG island regions" phrase and was not directed to

the "at least one nucleotide" phrase which is directly supported by the grammatical structure of this claim. However, in the sole interest of facilitating prosecution, Applicants have further amended this claim by substituting the phrase "within the CpG island regions encompassed by nucleotides 90 to 312, 836 to 1122, or 1331 to 1589" with the phrase "are mutated in a region selected from the group consisting of: a.) from about nucleotide –1968 to about –1746; b.) from about nucleotide –1232 to about –936; and c.) from about nucleotide –727 to about –470" to make it clear that these regions are the regions in which a mutation may reside, as well as to append the phrase "but not more than 10" after the "at least one nucleotide" phrase. Applicants further amended this claim by deleting the phrase "of SEQ ID NO:27 are mutated"; to append the term ", wherein" after the newly added "–470" term; to delete the phrase "such that " after the newly added ", wherein" term; and to substitute the phrase "the CpG islands contained therein" with the phrase "said regions" to make this claim for grammatically succinct. Applicants believe the Examiner's rejection of Claim 45 under 35 U.S.C. § 112 second paragraph, has been overcome in consideration of these amendments.

Applicants have amended Claim 46 to substitute the phrase "at least one CpG island region encompassed by nucleotides 90 to 312, 836 to 1122, and 1331 to 1589" with the phrase "a region selected from the group consisting of: a.) from about nucleotide –1968 to about –1746; b.) from about nucleotide –1232 to about –936; and c.) from about nucleotide –727 to about –470". Claim 46 was further amended to delete the phrase "of SEQ ID NO:27". Applicants believe the Examiner's rejection of Claim 46 under 35 U.S.C. § 112 second paragraph, has been overcome in consideration of these amendments.

c. The Examiner has rejected Claim 33 under 35 U.S.C. § 112, second paragraph alleging that it is indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. Specifically, the Examiner alleges

> "The phrase 'a polynucleotide that is at least 80% identical to amino acids 2-541' is unclear. It is unclear as how a polynucleotide should have identity to a polypeptide."

Applicants have amended Claim 33 to append the phrase "encoding a polypeptide" after the "polynucleotide" term. Applicants believe the Examiner's rejection of Claim 33 under 35 U.S.C. § 112 second paragraph, has been overcome in consideration of this amendment.

d. The Examiner has rejected Claims 30 to 31 under 35 U.S.C. § 112, second paragraph alleging that it is indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. Specifically, the Examiner alleges

Claims 30-31 recite the limitation "claims 30 refers back to said heterogonous nucleic acid sequence as a limitation. There is insufficient antecedent basis for this limitation in the claim. Similarly claim 31 refers to said heterogonous polypeptide and there is insufficient antecedent basis for said limitation.

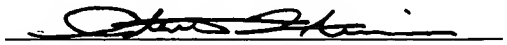
Applicants have amended Claim 30 to amend the dependency from "claim 31" to "claim 29". Applicants have also amended Claim 31 to amend the dependency from "claim 32" to "claim 30". Applicants believe the Examiner's rejection of Claim 33 under 35 U.S.C. § 112 second paragraph, has been overcome in consideration of this amendment.

Applicants believe that all of the Examiners rejections and objections have been overcome and that all of the pending claims before the Examiner are in condition for allowance. An early Office Action to that effect is, therefore, earnestly solicited.

If any fee is due in connection herewith not already accounted for, please charge such fee to Deposit Account No. 19-3880 of the undersigned. Furthermore, if any extension of time not already accounted for is required, such extension is hereby petitioned for, and it is requested that any fee due for said extension be charged to the above-stated Deposit Account.

Respectfully submitted,

Bristol-Myers Squibb Company
Patent Department
P.O. Box 4000
Princeton, NJ 08543-4000
(609) 252-5289



Stephen C. D'Amico
Agent for Applicants
Reg. No. 46,652

Date: January 9, 2006

Human Molecular Genetics, 2001, Vol. 10, No. 7 687–692

Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer

Stephen B. Baylin*, Manel Esteller, Michael R. Rountree, Kurtis E. Bachman, Kornel Schuebel and James G. Herman

The Johns Hopkins Comprehensive Cancer Center and Johns Hopkins Medical Institutions, Baltimore, MD 21231, USA

Received 10 January 2001 ; Accepted 15 January 2001

Gene function in cancer can be disrupted either through genetic alterations, which directly mutate or delete genes, or epigenetic alterations, which alter the heritable state of gene expression. The latter events are mediated by formation of transcriptionally repressive chromatin states around gene transcription start sites and an associated gain of methylation in normally unmethylated CpG islands in these regions. The genes affected include over half of the tumor suppressor genes that cause familial cancers when mutated in the germline; the selective advantage for genetic and epigenetic dysfunction in these genes is very similar. The aberrant methylation can begin very early in tumor progression and mediate most of the important pathway abnormalities in cancer including loss of cell cycle control, altered function of transcription factors, altered receptor function, disruption of normal cell–cell and cell–substratum interaction, inactivation of signal transduction pathways, loss of apoptotic signals and genetic instability. The active role of the aberrant methylation in transcriptional silencing of genes is becoming increasingly understood and involves a synergy between the methylation and histone deacetylase (HDAC) activity. This synergy can be mediated directly by HDAC interaction with DNA methylating enzymes and by recruitment through complexes involving methyl-cytosine binding proteins. In the translational arena, the promoter hypermethylation changes hold great promise as DNA tumor markers and their potentially reversible state creates a target for cancer therapeutic strategies involving gene reactivation.

INTRODUCTION

It is increasingly apparent that, in human cancers, heritable losses of gene function may be mediated as often by epigenetic, as by genetic, abnormalities (recent reviews in 1,2). The data, rather than fueling the old argument of whether cancer is an epigenetic or a genetic disease, in fact emphasize that synergy between these two processes drives tumor progression from the earliest to latest stages. Inclusion of epigenetic events in our concepts of how tumors evolve heightens our need to understand the basic nature of chromatin changes that set heritable states of gene function. Also, from a translational standpoint, it enriches the potential for molecular approaches to early tumor detection and profiling, and suggests new targets to consider for cancer prevention and therapeutic strategies. This review will highlight recent developments in all of the above arenas.

CHARACTERISTICS OF GENES HYPERMETHYLATED IN CANCER

The most widely studied epigenetic abnormality in tumorigenesis is the silencing of gene transcription associated with gains of DNA methylation in normally unmethylated gene promoter regions (1,2). Delineation of the specific genes affected by this process is receiving ever increasing emphasis, and searches by candidate gene and new genomic screening approaches (3–6) are a growing presence in cancer research.

The genes hypermethylated in cancer

We have recently reviewed the growing list of hypermethylated genes identified in human cancers (1) and stressed that over half the genes that cause familial forms of human cancer, when mutated in the germline, are included. These genes, such as *APC*, *BRCA-1*, *E-cadherin*, *LKB1*, *MLH1*, *p16*^{INK4a}, *Rb*, *VHL*, etc., can exhibit this change in non-familial cancers and the selective advantage for loss of gene function is very clear in several ways (1). First, for some of the genes, either mutations or the hypermethylation are observed in the same specific tumor types. Second, for several genes, tumor phenotypes predicted for loss of specific gene function accompany both the genetic and epigenetic changes. Third, the same disruption of key biological pathways can accompany either change.

The list of hypermethylated genes in cancer also includes many that are not fully documented tumor suppressors (1). Importantly, for some of these genes, the promoter methylation may be the only type of gene inactivation found in human cancer, since mutations for many of the genes are rare or have not been observed. The question then increasingly arises, as more strategies are employed to define hypermethylated loci in

*To whom correspondence should be addressed. Tel: +1 410 955 8506; Fax: +1 410 614 9884; Email: sbaylin@jhmi.edu

cancer, of whether promoter hypermethylation alone can pinpoint a gene as a tumor suppressor. In truth, the burden of proof for equating promoter hypermethylation with clonal selective advantage for tumor progression will always be more difficult than for gene mutations. Each hypermethylated gene identified must be carefully scrutinized for its role in the biology of a tumor type. This point is emphasized by recent observations that some genes may be altered as a group in tumors (6), suggesting that promoter hypermethylation might be viewed as a process or processes in cancer biology, much like loss of mismatch repair function (7). In this concept, the clonal presence of both microinstability and hypermethylation would reflect the importance of the 'process' itself to tumor development. Both critical and non-critical loci could be affected by either process and only those providing loss of key gene function would render selective advantage for tumor development.

Despite the above need for caution in interpreting the biological significance for hypermethylation of specific genes in cancer, both candidate gene and genomic scanning approaches (3,5,6,8) may serve to identify key genes for tumor development and other biologic processes. For example, we have identified hypermethylated CpG islands as a guide for cloning candidate tumor suppressor genes in a chromosomal area frequently deleted in cancer, but in which genetic alterations have not identified a resident tumor suppressor gene (9). The gene cloned, *HIC-1* (10), proved to be a new member of a family of zinc-finger transcription factors important to developmental processes, to harbor very frequent promoter hypermethylation in many tumor types (9–12) and to be up-regulated by the p53 protein (10,13). While its role in cancer is still being investigated, subsequent studies revealed that mice with homozygous knock-out of *Hic-1* die during embryogenesis, or perinatally, from a variety of developmental defects, some of which are found in a human genetic mental retardation syndrome (14). *HIC-1* resides in the obligate chromosome deletion associated with this disorder (14,15). Thus, screening for promoter hypermethylated loci in tumor DNA, and building databases designating the chromosome positions of these sites, could prove extremely valuable for defining genes important to cancer and other human diseases.

Timing of aberrant promoter methylation during tumor progression

Recent studies indicate that promoter hypermethylation is often an early event in tumor progression. The earliest stage appears to involve genes where promoter region hypermethylation increases in normal tissues as a function of aging. In fact, in the colon the genes with the highest incidence of promoter hypermethylation in tumors fall into this category and the age-related curves for the increase in hypermethylation and risk of colon cancer are remarkably similar (6,16,17). Interestingly, this group of genes does not include classic tumor suppressors (6,16,17) and many of the genes involved may not directly mediate tumor progression. However, some genes, such as the estrogen receptor where age-related hypermethylation in the colon was first described (16), may be important to the modulation of cell growth and differentiation in the colonic mucosa. Loss of function for such genes may then provide a permissive background for subsequent cellular events to foster tumor progression.

Promoter hypermethylation for genes known to play a critical role in tumorigenesis, and which are unmethylated in normal tissues at all ages, can also be found quite early in tumorigenesis. These early epigenetic alterations can, in fact, produce the early losses of cell cycle control, altered regulation of gene transcription factors, disruption of cell–cell and cell–substratum interaction and even multiple types of genetic instability, characteristic of human cancer (Fig. 1). For example, epigenetically mediated loss of $p16^{INK4a}$ function appears to help by-pass early mortality check-points critical to the onset of cellular immortality in experimental systems of carcinogenesis (18–23) and is also seen in early stages of naturally occurring tumors (24–27). Hypermethylation of the 'gatekeeper' gene for colon cancer, *APC*, critical for regulation of the β-catenin–TCF transcriptional pathway (28), has recently been recognized for a subset of colon cancers (29). Similarly, $p14^{ARF}$ gene hypermethylation may begin in pre-malignant colonic polyps (26) and thus determine how the metabolism of p53 and other proteins regulated by MDM2, the protein regulated by interaction with $p14^{ARF}$, is subsequently mediated during tumor progression (30,31). In terms of leading to specific genetic events fundamental to progression of tumors, hypermethylation of *MLH1* is the most frequent change associated with micro-satellite instability in colon, endometrial and gastric neoplasia (32–36). Also, early loss of expression of the repair enzyme gene, *O⁶–MGMT*, which guards against G→A mutations, accompanies promoter hypermethylation of this gene prior to stages where G→A mutations appear in the K-*ras* gene in colon cancer progression (37). Finally, in terms of cell–cell recognition events that can predispose to invasion, hypermethylation of the *E-cadherin* promoter frequently occurs in early stages of breast cancer (38).

Differences between loss of gene function via genetic versus epigenetic changes

While we have emphasized the similarities for selective advantage for the same gene when affected by promoter hypermethylation and mutations, there are some fundamental differences between such genetic and epigenetic events that are potentially very significant for tumor biology. First, when genetic events are responsible for disruption of both alleles in the classic two-hit paradigm for loss of tumor suppressor gene function, each event obviously produces a fixed level for loss of gene dosage. First genetic hits may potentially result in phenotypically functional haplo-insufficiency states (39). However, in most instances, the onset of selective cell advantage does not occur without the complete loss of gene dosage produced by the second hit. In contrast, the loss of gene transcription associated with aberrant promoter CpG island methylation is, experimentally (40), mediated by the density of methylation within the region. Recent data suggest that this density can increase over time of cell replications (41,42) and thus be associated with increasing degrees of transcription loss. A range of evolutionary gene dosage effects, rather than the immediate and fixed loss of function, could then evolve. Thus, gene function loss in association with aberrant promoter methylation may manifest in a more subtle selective advantage than gene mutations during tumor progression.

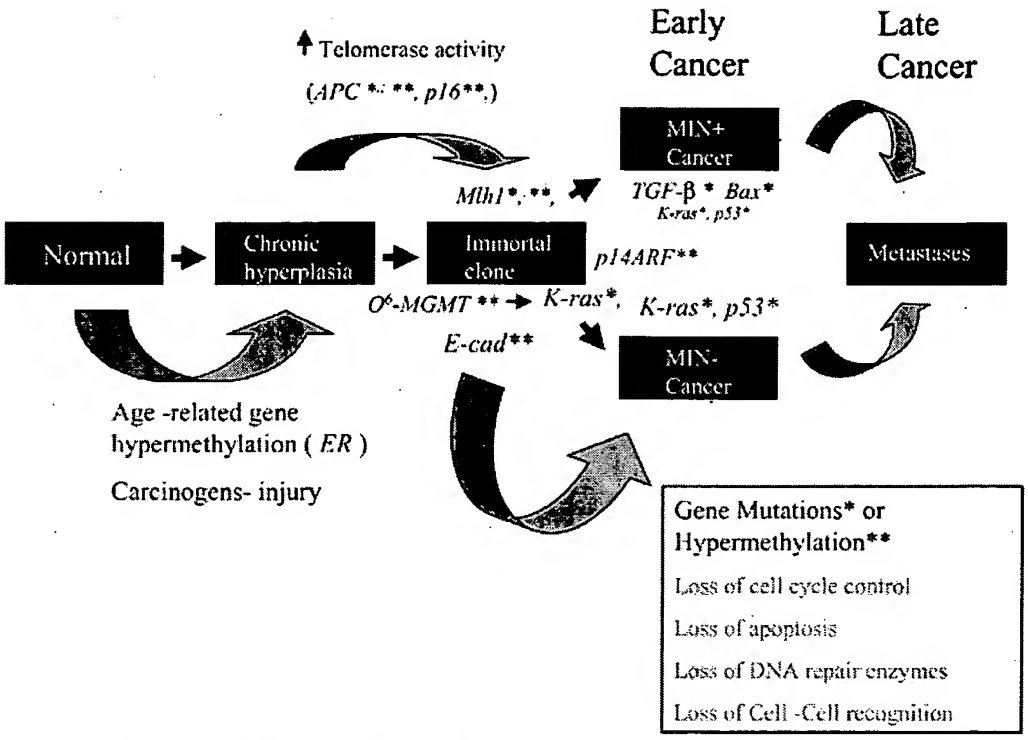


Figure 1. A model depicting the interaction between selected genetic and epigenetic events to drive the evolution of cancer. Although the model draws heavily on known events in colon cancer to illustrate key points, data from other epithelial tumors, and which may not be common in this specific tumor such as hypermethylation of *E-cadherin*, are included for completeness. In the tumor progression, a branch point is presented after emergence of the immortalized clone depicting colon tumors with (Min+) and without (Min–) the micro-satellite instability phenotype. For individual genes, genetic and epigenetic events known to be more common in DNA from either of these two types of tumors are depicted in larger letters. Specific aspects of the timing for gene inactivation events and predisposition of molecular changes fostered by these events are discussed in the text.

Second, while promoter hypermethylation and associated gene silencing generally remain very stable in cancer cells (43), these changes are, unlike mutations, potentially reversible. In fact, such epigenetic plasticity is an excellent candidate to mediate the dynamic heterogeneity of cell populations inherent to complex tumor traits such as metastasis. In this regard, most epithelial tumors are highly invasive, and the cells released can form metastatic foci, which grow within visceral organs. Loss of cellular E-cadherin, and the homotypic cell–cell contact that this protein mediates, highly facilitates initial tumor cell invasion in experimental systems. This protein is frequently lost in a cellularly heterogeneous pattern in native cancers (38). However, the ability of the invading cells to form metastatic foci within distant organs may require re-expression of E-cadherin to allow tumor cells to form cell aggregates necessary for survival in a foreign environment (44–46). These dynamics may explain why the heterogenous loss of E-cadherin is similar in both primary and metastatic tumor sites in the same patient (45,47,48).

Mutations in the E-cadherin gene, which would, of course, produce homogeneous cellular loss of function, are uncommon except in patients with familial gastric cancer who inherit germline *E-cadherin* mutations, and in lobular breast carcinomas, a relatively uncommon tumor cell type. Interestingly, although these tumors are highly invasive, the resultant metastases spread along membrane surfaces rather than growing within underlying organs. In contrast, the heterogeneous loss of E-cadherin gene expression so common in most major epithelial cancers occurs in the absence of coding region mutations for the gene and in association with a similarly heterogeneous pattern of promoter region hypermethylation (38). This promoter change is even allelically heterogenous in some long-term cultures of cancers. In such cultures, the most highly invasive cells have the heaviest promoter methylation (38). In contrast, when the cells are forced to grow as cellular aggregates, which mimic intra-organ metastatic foci, methylation decreases regionally in the *E-cadherin* promoter and the cells regain *E-cadherin* expression (38). Thus, environmentally controlled reversibility of aberrant promoter hypermethylation may, in some instances, play a major role in the cell population dynamics which foster key aspects of tumor behavior.

CHROMATIN FORMATION AS A MEDIATOR OF PATTERNS OF DNA METHYLATION AND GENE TRANSCRIPTION IN NORMAL AND NEOPLASTIC CELLS

One of the pressing issues in studying promoter DNA methylation in cancer is to understand how this DNA modification actually modulates gene expression. Central to this concept has been the pioneering work of Bird and co-workers (49–51) in

defining a family of proteins (MBDs) that preferentially bind to methyl-cytosines, and have an inherent capacity to inhibit gene transcription. Most recently, two of these MBDs, MeCP2 and MBD2 have been shown in addition to participate in protein complexes that recruit transcriptional co-repressors, chromatin remodeling proteins and histone deacetylases (52–54). Through such complexes, sites of DNA methylation could then target the formation of chromatin, including the deacetylated state of histones, which is typical for transcriptionally repressive domains (49–54).

The above dynamics of chromatin formation suggest that DNA methylation and histone deacetylation might work in concert to silence hypermethylated genes in tumors. Our own studies have, indeed, revealed that multiple such hypermethylated genes will not re-express in cancer cells with inhibition of HDAC activity alone, by agents such as trichostatin (TSA). However, this drug becomes effective for such purposes if minimal de-methylation of the involved promoters is first achieved with low doses of demethylating agents such as 5-aza-cytidine (55). This paradigm is characteristic of other densely methylated genes including the mutated, hypermethylated, fragile-X gene (56).

What is the mechanism underlying the above sensitization by demethylation of gene re-expression to TSA? Certainly, the loss of methylation might reduce the numbers of MBD complexes at a given locus leaving reduced amounts of HDAC to be inhibited by TSA. Also, loss of the transcription repression complexes might favor re-association with the gene promoters of transcription activation complexes with co-activators which possess histone acetylase activity. However, recent advances in our understanding of complexes and activities associated with the DNA methylating machinery itself may be important for full understanding of the process. During the past year, three groups have revealed that the most abundant of the mammalian DNA methyltransferases (DNMTs), DNMT1, can directly bind HDACs (57–59). In addition, the N-terminus of this protein can bind transcriptional co-repressors and directly suppress, in a partially HDAC-dependent manner, gene transcription independent of the C-terminal methylation catalytic site (59). Thus, demethylation of gene promoters, and the activity of DNMT inhibitors such as 5-azacytidine, could have complex effects on localization and activity of HDACs that had not been previously contemplated. It will now be essential to identify whether these DNMT1 complexes associate with specific gene loci in normal and neoplastic cells.

In addition to the role of DNMT1 in normal and malignant cells, we must now consider two additional biologically active mammalian DNMTs, 3a and 3b. Like DNMT1, these two proteins are essential for mammalian development (60,61), and studies of mouse development suggest that DNMT3a and 3b catalyze, principally, *de novo* patterns of methylation that arise during this period (60,61). However, the function of these latter two proteins, if any, in adult normal and neoplastic cells, is not yet known.

With respect to cancer cells, circumstantial evidence has suggested a role for DNMT1 in aberrant promoter methylation. Forced expression of this enzyme can yield, with multiple cell passages, hypermethylation of formerly unmethylated promoter region CpG islands (41). However, our group has recently participated in the surprising finding that bi-allelic knockout of *DNMT1* in cultured human colon cancer cells, while abolishing most of the DNA methylating activity in the cells, did not result in loss of aberrant methylation in multiple hypermethylated genes examined (62). While this ability of the cells to maintain such methylation may be a product of the selection of cells especially equipped to function without DNMT1, the results stress that other DNMTs could participate in the abnormal methylation patterns seen in cancer cells. Certainly, DNMTs 3a and 3b become key candidates. These enzymes, like DNMT1, appear to be modestly up-regulated in tumor cells (63). Perhaps, multiple DNMTs may collaborate to produce and maintain DNA methylation patterns in normal and neoplastic cells. Investigating the co-ordinate role of such a collaboration, especially with respect to the specific genomic loci hypermethylated in tumors, should yield much fruitful information over the next few years.

TRANSLATIONAL IMPLICATIONS OF PROMOTER REGION HYPERMETHYLATION IN CANCER

Even as the basic mechanisms underlying promoter hypermethylation and gene silencing are still being dissected, at least two important translational implications of the DNA change are already receiving increasing attention. The first involves new strategies for early tumor detection and prognosis prediction and the second involves prevention and treatment strategies that rely on the potentially reversible nature of epigenetically mediated altered gene function.

Promoter region hypermethylation—the ideal tumor marker?

In tumors from different patients, even of the same histologic type, mutations that disrupt gene function often vary in genomic position over a wide region. In contrast, the position of CpG island promoter methylation is constant within an individual gene. Potentially then, for all patients, a single primer strategy can be used to detect tumor-specific methylation changes in a given gene by recently developed sensitive, methylation-specific, PCR procedures (64). Such assays could be applied to DNA obtained from distal sites such as serum, urine or sputum, even without knowing the methylation status of the marker directly in primary tumor DNA. This detection is facilitated by the fact that the PCR signal will be a positive, rather than a negative one, such as loss of allelic heterozygosity. These characteristics of promoter hypermethylation, coupled with the facts that this change often occurs early in tumor progression for the genes studied, and that all tumors appear to have one or more hypermethylated loci when panels of these markers are examined, renders this change potentially valuable as a DNA marker for sensitive early tumor detection.

Extremely encouraging support for the above hypothesis is being obtained in early, small proof of principal studies. Hypermethylated promoter loci have been detected with high specificity in serum DNA from patients with lung, hepatic, breast and other tumors (65–67). Particularly exciting are recent studies of lung cancer patients where one of two hypermethylation loci were always positive in both tumor and sputum DNA, and were detected in the latter samples up to 3 years prior to clinical diagnosis (68). Continued findings of this kind will justify larger and larger population studies of

specific hypermethylation marker panels in individuals at high risk for specific tumor types.

Gene promoter hypermethylation changes may also provide markers for predicting specific aspects of tumor behavior. For example, hypermethylation of a gene for which loss of function has been correlated to high metastatic potential in an animal model correlates with lung cancer tumor virulence in patients (69). In a recent study of brain tumors by our own group, the presence of hypermethylation of the promoter of a gene that encodes for a DNA repair protein highly correlated with response of the cancers to a chemotherapy agent that works through alkylation damage of DNA (70). Thus, patterns of hypermethylated genes in specific cancer types may provide one means for building DNA profiles that predict key aspects of tumor behavior in individual patients.

The potentially reversible nature of loss of gene function associated with aberrant promoter methylation—a therapeutic target for cancer?

Unlike genetic changes in cancer, as we have previously discussed, epigenetic changes are potentially reversible. Reactivation of the silencing associated with promoter methylation for critical genes, such as *p16*, would be a highly desirable goal for reversing many aspects of the cancer cell phenotype. Indeed, even before the methylation changes in cancer were known to involve such gene promoters, demethylating agents such as 5-Aza C were being tried as chemotherapy agents. Several years of study have documented some efficacy in hematopoietic malignancies, especially studies by Lubbert (71) and Momparler *et al.* (72). However, the mechanisms underlying the effectiveness of such drugs in the therapeutic setting have not been closely investigated. Also, considerable toxicity to areas such as the bone marrow (72) has been observed and can be limiting. Much of this toxicity could be unrelated to effects on methylation, such as production of DNA damage through creation of actual adducts between DNA and DNA-methyltransferases (73,74).

The recognition of promoter hypermethylation-associated gene silencing in cancer has spurred considerable new interest in gaining more specificity of drugs like 5-Aza C for DNA demethylation. Also, the recent findings that initial very modest de-methylation of such gene promoters can sensitize hypermethylated genes to reactivation with HDAC inhibitors (55) is already receiving attention in the clinic. A Phase I trial of this concept for all tumor types is actually underway at our institution using 5-Aza -C and the HDAC inhibitor, phenylbutyrate. The initial aim is to reduce the doses of 5-Aza -C for the drug combination, below levels that result in systemic toxicity, and to monitor the potential reactivation of key genes. Any observed clinical efficacy in studies of this type will help fuel the current interest in not only the basic biology of epigenetically mediated gene changes in cancer, but the potential for reversing these in patients for therapeutic purposes.

REFERENCES

1. Baylin, S.B. and Herman, J.G. (2000) DNA hypermethylation in tumorigenesis: epigenetics joins genetics. *Trends Genet.*, **16**, 168–174.
2. Jones, P.A. and Laird, P.W. (1999) Cancer epigenetics comes of age. *Nature Genet.*, **21**, 163–167.
3. Costello, J.F., Fruhwald, M.C., Smiraglia, D.J. *et al.* (2000) Aberrant CpG-island methylation has non-random and tumor-type-specific patterns. *Nature Genet.*, **24**, 132–138.
4. Huang, T.H., Perry, M.R. and Laux, D.E. (1999) Methylation profiling of CpG islands in human breast cancer cells. *Hum. Mol. Genet.*, **8**, 459–470.
5. Toyota, M., Ho, C., Ahuja, N. *et al.* (1999) Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification. *Cancer Res.*, **59**, 2307–2312.
6. Toyota, M., Ahuja, N., Ohe-Toyota, M., Herman, J.G., Baylin, S.B. and Issa, J.P. (1999) CpG island methylator phenotype in colorectal cancer. *Proc. Natl Acad. Sci. USA*, **96**, 8681–8686.
7. Baylin, S.B., Belinsky, S.A. and Herman, J.G. (2000) Aberrant methylation of gene promoters in cancer—concepts, misconcepts and promise. *J. Natl Cancer Inst.*, **92**, 1460–1461.
8. Plass, C., Yu, F., Yu, L. *et al.* (1999) Restriction landmark genome scanning for aberrant methylation in primary refractory and relapsed acute myeloid leukemia; involvement of the WIT-1 gene. *Oncogene*, **18**, 3159–3165.
9. Makos, M., Nelkin, B.D., Lerman, M.I., Latif, F., Zbar, B. and Baylin, S.B. (1992) Distinct hypermethylation patterns occur at altered chromosome loci in human lung and colon cancer. *Proc. Natl Acad. Sci. USA*, **89**, 1929–1933.
10. Wales, M.M., Biel, M.A., el Deiry, W. *et al.* (1995) p53 activates expression of HIC-1, a new candidate tumour suppressor gene on 17p13.3. *Nature Med.*, **1**, 570–577.
11. Makos, M., Nelkin, B.D., Reiter, R.E. *et al.* (1993) Regional DNA hypermethylation at D17S5 precedes 17p structural changes in the progression of renal tumors. *Cancer Res.*, **53**, 2719–2722.
12. Fujii, H., Biel, M.A., Zhou, W., Weitzman, S.A., Baylin, S.B. and Gabrielson, E. (1998) Methylation of the HIC-1 candidate tumor suppressor gene in human breast cancer. *Oncogene*, **16**, 2159–2164.
13. Guerardel, C., Deltour, S., Pinte, S. *et al.* (2001) Identification in the human candidate tumor suppressor gene HIC-1 of a new major alternative TATA-less promoter positively regulated by p53. *J. Biol. Chem.*, **275**, 307–308.
14. Carter, M.G., Johns, M.A., Zeng, X. *et al.* (2000) Mice deficient in the candidate tumor suppressor gene Hic1 exhibit developmental defects of structures affected in the Miller–Dieker syndrome. *Hum. Mol. Genet.*, **9**, 413–419.
15. Hirotsune, S., Pack, S.D., Chong, S.S. *et al.* (1997) Genomic organization of the murine Miller–Dieker/lissencephaly region: conservation of linkage with the human region. *Genome Res.*, **7**, 625–634.
16. Issa, J.P., Ottaviano, Y.L., Celano, P., Hamilton, S.R., Davidson, N.E. and Baylin, S.B. (1994) Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. *Nature Genet.*, **7**, 536–540.
17. Ahuja, N., Li, Q., Mohan, A.L., Baylin, S.B. and Issa, J.P. (1998) Aging and DNA methylation in colorectal mucosa and cancer. *Cancer Res.*, **58**, 5489–5494.
18. Foster, S.A., Wong, D.J., Barrett, M.T. and Galloway, D.A. (1998) Inactivation of p16 in human mammary epithelial cells by CpG island methylation. *Mol. Cell. Biol.*, **18**, 1793–1801.
19. Loughran, O., Malliri, A., Owens, D. *et al.* (1996) Association of CDKN2A/p16INK4A with human head and neck keratinocyte replicative senescence: relationship of dysfunction to immortality and neoplasia. *Oncogene*, **13**, 561–568.
20. Brenner, A.J., Stampfer, M.R. and Aldaz, C.M. (1998) Increased p16 expression with first senescence arrest in human mammary epithelial cells and extended growth capacity with p16 inactivation. *Oncogene*, **17**, 199–205.
21. Vogt, M., Haggblom, C., Yeargin, J., Christiansen-Weber, T. and Haas, M. (1998) Independent induction of senescence by p16INK4a and p21CIP1 in spontaneously immortalized human fibroblasts. *Cell Growth Differ.*, **9**, 139–146.
22. Huschtscha, L.I., Noble, J.R., Neumann, A.A. *et al.* (1998) Loss of p16INK4 expression by methylation is associated with lifespan extension of human mammary epithelial cells. *Cancer Res.*, **58**, 3508–3512.
23. Jarrard, D.F., Sarkar, S., Shi, Y. *et al.* (1999) p16/pRb pathway alterations are required for bypassing senescence in human prostate epithelial cells. *Cancer Res.*, **59**, 2957–2964.
24. Wong, D.J., Barrett, M.T., Stoger, R., Emond, M.J. and Reid, B.J. (1997) p16INK4a promoter is hypermethylated at a high frequency in esophageal adenocarcinomas. *Cancer Res.*, **57**, 2619–2622.
25. Belinsky, S.A., Nikula, K.J., Palmisano, W.A. *et al.* (1998) Aberrant methylation of p16(INK4a) is an early event in lung cancer and a potential biomarker for early diagnosis. *Proc. Natl Acad. Sci. USA*, **95**, 11891–11896.
26. Esteller, M., Tortola, S., Toyota, M. *et al.* (2000) Hypermethylation-associated inactivation of p14 (ARF) is independent of p16(INK4a) methylation and p53 mutational status. *Cancer Res.*, **60**, 129–133.
27. Nuovo, G.J., Plaia, T.W., Belinsky, S.A., Baylin, S.B. and Herman, J.G. (1999) *In situ* detection of the hypermethylation-induced inactivation of

the p16 gene as an early event in oncogenesis. *Proc. Natl Acad. Sci. USA*, **96**, 12754–12759.
28. Kinzler, K.W. and Vogelstein, B. (1997) Cancer-susceptibility genes. Gatekeepers and caretakers. *Nature*, **386**, 761–763.
29. Esteller, M., Sparks, A., Toyota, M. *et al.* (2000) Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. *Cancer Res.*, **60**, 4366–4371.
30. Zhang, Y., Xiong, Y. and Yarbrough, W.G. (1998) ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell*, **92**, 725–734.
31. Stott, F.J., Bates, S., James, M.C. *et al.* (1998) The alternative product from the human CDKN2A locus, p14 (ARF), participates in a regulatory feedback loop with p53 and MDM2. *EMBO J.*, **17**, 5001–5014.
32. Kane, M.F., Loda, M., Gaida, G.M. *et al.* (1997) Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Res.*, **57**, 808–811.
33. Herman, J.G., Umar, A., Polyak, K. *et al.* (1998) Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc. Natl Acad. Sci. USA*, **95**, 6870–6875.
34. Cunningham, J.M., Christensen, E.R., Tester, D.J. *et al.* (1998) Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. *Cancer Res.*, **58**, 3455–3460.
35. Esteller, M., Levine, R., Baylin, S.B., Ellenson, L.H. and Herman, J.G. (1998) MLH1 promoter hypermethylation is associated with the microsatellite instability phenotype in sporadic endometrial carcinomas. *Oncogene*, **17**, 2413–2417.
36. Simpkins, S.B., Bocker, T., Swisher, E.M. *et al.* (1999) MLH1 promoter methylation and gene silencing is the primary cause of microsatellite instability in sporadic endometrial cancers. *Hum. Mol. Genet.*, **8**, 661–666.
37. Esteller, M., Toyota, M., Sanchez-Cespedes, M. *et al.* (2000) Inactivation of the DNA repair gene O^6-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-*ras* in colorectal tumorigenesis. *Cancer Res.*, **60**, 2368–2371.
38. Graff, J.R., Gabrielson, E., Fujii, H., Baylin, S.B. and Herman, J.G. (2000) Methylation patterns of the E-cadherin 5′ CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. *J. Biol. Chem.*, **275**, 2727–2732.
39. Pietenpol, J.A., Bohlander, S.K., Sato, Y. *et al.* (1995) Assignment of the human p27Kip1 gene to 12p13 and its analysis in leukemias. *Cancer Res.*, **55**, 1206–1210.
40. Hsieh, C.L. (1994) Dependence of transcriptional repression on CpG methylation density. *Mol. Cell. Biol.*, **14**, 5487–5494.
41. Vertino, P.M., Yen, R.W., Gao, J. and Baylin, S.B. (1996) *De novo* methylation of CpG island sequences in human fibroblasts overexpressing DNA (cytosine-5-)-methyltransferase. *Mol. Cell. Biol.*, **16**, 4555–4565.
42. Graff, J.R., Herman, J.G., Myohanen, S., Baylin, S.B. and Vertino, P.M. (1997) Mapping patterns of CpG island methylation in normal and neoplastic cells implicates both upstream and downstream regions in *de novo* methylation. *J. Biol. Chem.*, **272**, 22322–22329.
43. Myohanen, S.K., Baylin, S.B. and Herman, J.G. (1998) Hypermethylation can selectively silence individual p16ink4A alleles in neoplasia. *Cancer Res.*, **58**, 591–593.
44. Takeichi, M. (1991) Cadherin cell adhesion receptors as a morphogenetic regulator. *Science*, **251**, 1451–1455.
45. Mareel, M., Bracke, M. and Van Roy, F. (1995) Cancer metastasis: negative regulation by an invasion-suppressor complex. *Cancer Detect. Prev.*, **19**, 451–464.
46. St Croix, B. and Kerbel, R.S. (1997) Cell adhesion and drug resistance in cancer. *Curr. Opin. Oncol.*, **9**, 549–556.
47. Moll, R., Mitze, M., Frixen, U.H. and Birchmeier, W. (1993) Differential loss of E-cadherin expression in infiltrating ductal and lobular breast carcinomas. *Am. J. Pathol.*, **143**, 1731–1742.
48. Siitonen, S.M., Kononen, J.T., Helin, H.J., Rantala, I.S., Holli, K.A. and Isola, J.J. (1996) Reduced E-cadherin expression is associated with invasiveness and unfavorable prognosis in breast cancer. *Am. J. Clin. Pathol.*, **105**, 394–402.
49. Robertson, K.D. and Jones, P.A. (2000) DNA methylation in health and disease. *Nature Rev. Genet.*, **1**, 11–19.
50. Bird, A.P. and Wolffe, A.P. (1999) Methylation-induced repression—belts, braces and chromatin. *Cell*, **99**, 451–454.
51. Ng, H.H. and Bird, A. (1999) DNA methylation and chromatin modification. *Curr. Opin. Genet. Dev.*, **9**, 158–163.
52. Jones, P.L., Veenstra, G.J., Wade, P.A. *et al.* (1998) Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nature Genet.*, **19**, 187–191.
53. Nan, X., Ng, H.H., Johnson, C.A. *et al.* (1998) Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature*, **393**, 386–389.
54. Wade, P.A., Gegonne, A., Jones, P.L., Ballestar, E., Aubry, F. and Wolffe, A.P. (1999) Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. *Nature Genet.*, **23**, 62–66.
55. Cameron, E.E., Bachman, K.E., Myohanen, S., Herman, J.G. and Baylin, S.B. (1999) Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nature Genet.*, **21**, 103–107.
56. Chiurazzi, P., Pomponi, M.G., Pietrobono, R., Bakker, C.E., Neri, G. and Oostra, B.A. (1999) Synergistic effect of histone hyperacetylation and DNA demethylation in the reactivation of the FMR1 gene. *Hum. Mol. Genet.*, **8**, 2317–2323.
57. Fuks, F., Burgers, W.A., Brehm, A., Hughes-Davies, L. and Kouzarides, T. (2000) DNA methyltransferase Dnmt1 associates with histone deacetylase activity. *Nature Genet.*, **24**, 88–91.
58. Robertson, K.D., Ait-Si-Ali, S., Yokochi, T., Wade, P.A., Jones, P.L. and Wolffe, A.P. (2000) DNMT1 forms a complex with rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nature Genet.*, **25**, 338–342.
59. Rountree, M.R., Bachman, K.E. and Baylin, S.B. (2000) DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. *Nature Genet.*, **25**, 269–277.
60. Okano, M., Bell, D.W., Haber, D.A. and Li, E. (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation and mammalian development. *Cell*, **99**, 247–257.
61. Okano, M., Xie, S. and Li, E. (1998) Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nature Genet.*, **19**, 219–220.
62. Rhee, I., Jair, K.W., Yen, R.W. *et al.* (2000) CpG methylation is maintained in human cancer cells lacking DNMT1. *Nature*, **404**, 1003–1007.
63. Robertson, K.D., Uzvolgyi, E., Liang, G. *et al.* (1999) The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. *Nucleic Acids Res.*, **27**, 2291–2298.
64. Herman, J.G., Graff, J.R., Myohanen, S., Nelkin, B.D. and Baylin, S.B. (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl Acad. Sci. USA*, **93**, 9821–9826.
65. Wong, I.H., Lo, Y.M., Zhang, J. *et al.* (1999) Detection of aberrant p16 methylation in the plasma and serum of liver cancer patients. *Cancer Res.*, **59**, 71–73.
66. Esteller, M., Sanchez-Cespedes, M., Rosell, R., Sidransky, D., Baylin, S.B. and Herman, J.G. (1999) Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. *Cancer Res.*, **59**, 67–70.
67. Sanchez-Cespedes, M., Esteller, M., Wu, L. *et al.* (2000) Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. *Cancer Res.*, **60**, 892–895.
68. Palmisano, W.A., Divine, K.K., Saccomanno, G. *et al.* (2000) Predicting lung cancer by detecting aberrant promoter methylation in sputum. *Cancer Res.*, **60**, 5954–5958.
69. Tang, X., Khuri, F.R., Lee, J.J. *et al.* (2000) Hypermethylation of the death-associated protein (DAP) kinase promoter and aggressiveness in stage I non-small-cell lung cancer. *J. Natl Cancer Inst.*, **92**, 1511–1516.
70. Esteller, M., Garcia-Foncillas, J., Andion, E. *et al.* (2000) Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N. Engl. J. Med.*, **343**, 1350–1354.
71. Lubbert, M. (2000) DNA methylation inhibitors in the treatment of leukemias, myelodysplastic syndromes and hemoglobinopathies: clinical results and possible mechanisms of action. *Curr. Top. Microbiol. Immunol.*, **249**, 135–164.
72. Momparler, R.L., Cote, S. and Eliopoulos, N. (1997) Pharmacological approach for optimization of the dose schedule of 5-aza-2′-deoxycytidine (Decitabine) for the therapy of leukemia. *Leukemia*, **11** (suppl.), S1–S6.
73. Ferguson, A.T., Vertino, P.M., Spitzner, J.R., Baylin, S.B., Muller, M.T. and Davidson, N.E. (1997) Role of estrogen receptor gene demethylation and DNA methyltransferase.DNA adduct formation in 5-aza-2′deoxycytidine-induced cytotoxicity in human breast cancer cells. *J. Biol. Chem.*, **272**, 32260–32266.
74. Jackson-Grusby, L., Laird, P.W., Magge, S.N., Moeller, B.J. and Jaenisch, R. (1997) Mutagenicity of 5-aza-2′-deoxycytidine is mediated by the mammalian DNA methyltransferase. *Proc. Natl Acad. Sci. USA*, **94**, 4681–4685.

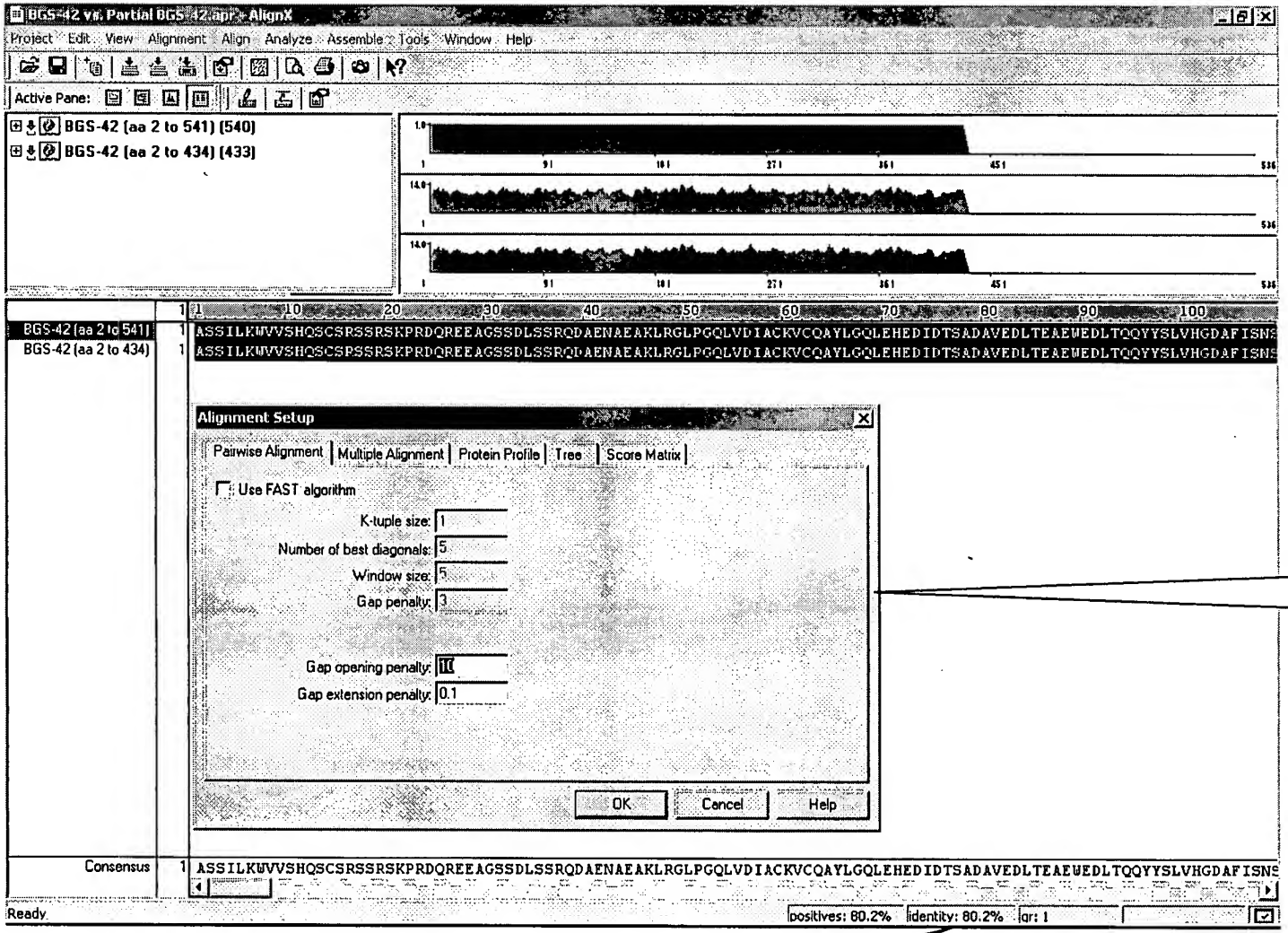
Exhibit A

```
                                          1                                                  50
Partial BGS-42 (SEQ ID NO:4)     (1) --------------------------------------------------
        BGS-42 (SEQ ID NO:2)     (1) MASSILKWVVSHQSCSRSSRSKPRDQREEAGSSDLSSRQDAENAEAKLRG

                                         51                                                 100
Partial BGS-42 (SEQ ID NO:4)     (1) --------------------------------------------------
        BGS-42 (SEQ ID NO:2)    (51) LPGQLVDIACKVCQAYLGQLEHEDIDTSADAVEDLTEAEWEDLTQQYYSL

                                        101                                                 150
Partial BGS-42 (SEQ ID NO:4)     (1) --------------------------------IDGLRNIWIIKPAAKSRG
        BGS-42 (SEQ ID NO:2)   (101) VHGDAFISNSRNYFSQCQALLNRITSVNPQTDIDGLRNIWIIKPAAKSRG

                                        151                                                 200
Partial BGS-42 (SEQ ID NO:4)    (19) RDIVCMDRVEEILELAAADHPLSRDNKWVVQKYIETPLLICDTKFDIRQW
        BGS-42 (SEQ ID NO:2)   (151) RDIVCMDRVEEILELAAADHPLSRDNKWVVQKYIETPLLICDTKFDIRQW

                                        201                                                 250
Partial BGS-42 (SEQ ID NO:4)    (69) FLVTDWNPLTIWFYKESYLRFSTQRFSLDKLDSAIHLCNNAVQKYLKNDV
        BGS-42 (SEQ ID NO:2)   (201) FLVTDWNPLTIWFYKESYLRFSTQRFSLDKLDSAIHLCNNAVQKYLKNDV

                                        251                                                 300
Partial BGS-42 (SEQ ID NO:4)   (119) GRSPLLPAHNMWTSTRFQEYLQRQGRGAVWGSVIYPSMKKAIAHAMKVAQ
        BGS-42 (SEQ ID NO:2)   (251) GRSPLLPAHNMWTSTRFQEYLQRQGRGAVWGSVIYPSMKKAIAHAMKVAQ

                                        301                                                 350
Partial BGS-42 (SEQ ID NO:4)   (169) DHVEPRKNSFELYGADFVLGRDFRPWLIEINSSPTMHPSTPVTAQLCAQV
        BGS-42 (SEQ ID NO:2)   (301) DHVEPRKNSFELYGADFVLGRDFRPWLIEINSSPTMHPSTPVTAQLCAQV

                                        351                                                 400
Partial BGS-42 (SEQ ID NO:4)   (219) QEDTIKVAVDRSCDIGNFELLWRQ------------------------
        BGS-42 (SEQ ID NO:2)   (351) QEDTIKVAVDRSCDIGNFELLWRQPVVEPPPFSGSDLCVAGVSVRRARRQ

                                        401                                                 450
Partial BGS-42 (SEQ ID NO:4)   (243) --------------------------------------------------
        BGS-42 (SEQ ID NO:2)   (401) VLPVCNLKASASLLDAQPLKARGPSAMPDPAQGPPSPALQRDLGLKEEKG

                                        451                                                 500
Partial BGS-42 (SEQ ID NO:4)   (243) --------------------------------------------------
        BGS-42 (SEQ ID NO:2)   (451) LPLALLAPLRGAAESGGAAQPTRTKAAGKVELPACPCRHVDSQAPNTGVP

                                        501                                 541
Partial BGS-42 (SEQ ID NO:4)   (243) ------------------------------------
        BGS-42 (SEQ ID NO:2)   (501) VAQPAKSWDPNQLNAHPLEPVLRGLKTAEGALRPPPGGKGS
```

Exhibit B-1

```
                                    1                                                  50
BGS-42 (aa 2 to 541)      (1)  ASSILKWVVSHQSCSRSSRSKPRDQREEAGSSDLSSRQDAENAEAKLRGL
BGS-42 (aa 2 to 434)      (1)  ASSILKWVVSHQSCSRSSRSKPRDQREEAGSSDLSSRQDAENAEAKLRGL

                                   51                                                 100
BGS-42 (aa 2 to 541)     (51)  PGQLVDIACKVCQAYLGQLEHEDIDTSADAVEDLTEAEWEDLTQQYYSLV
BGS-42 (aa 2 to 434)     (51)  PGQLVDIACKVCQAYLGQLEHEDIDTSADAVEDLTEAEWEDLTQQYYSLV

                                  101                                                 150
BGS-42 (aa 2 to 541)    (101)  HGDAFISNSRNYFSQCQALLNRITSVNPQTDIDGLRNIWIIKPAAKSRGR
BGS-42 (aa 2 to 434)    (101)  HGDAFISNSRNYFSQCQALLNRITSVNPQTDIDGLRNIWIIKPAAKSRGR

                                  151                                                 200
BGS-42 (aa 2 to 541)    (151)  DIVCMDRVEEILELAAADHPLSRDNKWVVQKYIETPLLICDTKFDIRQWF
BGS-42 (aa 2 to 434)    (151)  DIVCMDRVEEILELAAADHPLSRDNKWVVQKYIETPLLICDTKFDIRQWF

                                  201                                                 250
BGS-42 (aa 2 to 541)    (201)  LVTDWNPLTIWFYKESYLRFSTQRFSLDKLDSAIHLCNNAVQKYLKNDVG
BGS-42 (aa 2 to 434)    (201)  LVTDWNPLTIWFYKESYLRFSTQRFSLDKLDSAIHLCNNAVQKYLKNDVG

                                  251                                                 300
BGS-42 (aa 2 to 541)    (251)  RSPLLPAHNMWTSTRFQEYLQRQGRGAVWGSVIYPSMKKAIAHAMKVAQD
BGS-42 (aa 2 to 434)    (251)  RSPLLPAHNMWTSTRFQEYLQRQGRGAVWGSVIYPSMKKAIAHAMKVAQD

                                  301                                                 350
BGS-42 (aa 2 to 541)    (301)  HVEPRKNSFELYGADFVLGRDFRPWLIEINSSPTMHPSTPVTAQLCAQVQ
BGS-42 (aa 2 to 434)    (301)  HVEPRKNSFELYGADFVLGRDFRPWLIEINSSPTMHPSTPVTAQLCAQVQ

                                  351                                                 400
BGS-42 (aa 2 to 541)    (351)  EDTIKVAVDRSCDIGNFELLWRQPVVEPPPFSGSDLCVAGVSVRRARRQV
BGS-42 (aa 2 to 434)    (351)  EDTIKVAVDRSCDIGNFELLWRQPVVEPPPFSGSDLCVAGVSVRRARRQV

                                  401                                                 450
BGS-42 (aa 2 to 541)    (401)  LPVCNLKASASLLDAQPLKARGPSAMPDPAQGPPSPALQRDLGLKEEKGL
BGS-42 (aa 2 to 434)    (401)  LPVCNLKASASLLDAQPLKARGPSAMPDPAQGP----------------

                                  451                                                 500
BGS-42 (aa 2 to 541)    (451)  PLALLAPLRGAAESGGAAQPTRTKAAGKVELPACPCRHVDSQAPNTGVPV
BGS-42 (aa 2 to 434)    (434)  --------------------------------------------------

                                  501                                  540
BGS-42 (aa 2 to 541)    (501)  AQPAKSWDPNQLNAHPLEPVLRGLKTAEGALRPPPGGKGS
BGS-42 (aa 2 to 434)    (434)  ---------------------------------------
```

Exhibit B-2



CLUSTALW default parameters

Percent Identity